Hepatitis E virus as a cause of acute hepatitis acquired in Switzerland

Fraga, Montserrat ; Doerig, Christopher ; Moulin, Hervé ; Bihl, Florian ; Brunner, Felix ; Müllhaupt, Beat ; Ripellino, Paolo ; Semela, David ; Stickel, Felix ; Terziroli Beretta-Piccoli, Benedetta ; Aubert, Vincent ; Telenti, Amalio ; Greub, Gilbert ; Sahli, Roland ; Moradpour, Darius

Abstract: BACKGROUND: Autochthonous hepatitis E is increasingly recognized as zoonotic infection in western countries. Serological assays have varying sensitivity and specificity. METHODS: We implemented molecular testing to identify and characterize acute hepatitis E acquired in Switzerland. RESULTS: Ninety-three cases of mostly symptomatic acute hepatitis E acquired in Switzerland were documented by PCR between November 2011 and December 2016. Median HEV RNA was 7.5 x 104 IU/mL (range, 5.3 to 4.7 x 107 IU/mL). HEV genotyping was successful in 78 patients, revealing genotype 3 in 75 and genotype 4 in three patients. Phylogenetic analyses revealed a few limited geographical and temporal clusters. Of the 91 patients with available anti-HEV IgM serology, four were negative; three of these were also IgG-negative, likely as a result of immunosuppression, and one was IgG-positive, a constellation compatible with HEV reinfection. Median age of the patients was 58 years (range, 20-80 years); 71 (76.3%) were men and 49 of these (69.0%) were 50 years old. The clinical course was particularly severe in patients with underlying chronic liver disease, with fatal outcome in two patients. Six patients (6.5%) presented with neuralgic amyotrophy. CONCLUSIONS: Nucleic acid-based diagnosis reveals HEV as a relevant cause of acute hepatitis in Switzerland. Middle-aged and elderly men constitute the majority of symptomatic patients. Testing for HEV should be included early in the diagnostic workup of acute hepatitis and of neuralgic amyotrophy, a typical extrahepatic manifestation of HEV genotype 3 infection.

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Hepatitis E Virus as a Cause of Acute Hepatitis Acquired in Switzerland

Montserrat Fraga[1]*, Christopher Doerig[1], Hervé Moulin[2], Florian Bihl[3], Felix Brunner[4], Beat Müllhaupt[5], Paolo Ripellino[6], David Semela[7], Felix Stickel[5], Benedetta Terziroli Beretta-Piccoli[8], Vincent Aubert[9], Amalio Telenti[2#], Gilbert Greub[2], Roland Sahli[2]* and Darius Moradpour[1]*

[1]Division of Gastroenterology and Hepatology, [2]Institute of Microbiology as well as [9]Division of Immunology and Allergy, University Hospital Lausanne, [3]Hepatology Service, Ente Ospedaliero Cantonale Ticino, Lugano and University Hospital Geneva, [4]Hepatology, University Clinic for Visceral Surgery and Medicine, University Hospital Berne, [5]Division of Gastroenterology and Hepatology, University Hospital Zurich, [6]Neurocenter of Southern Switzerland, Lugano, [7]Division of Gastro-enterology and Hepatology, Cantonal Hospital St. Gallen, [8]Epatocentro Ticino, Lugano, Switzerland

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*Correspondence. Montserrat Fraga, M.D., Roland Sahli, Ph.D. or Darius Moradpour, M.D., Centre Hospitalier Universitaire Vaudois, University of Lausanne, Rue du Bugnon 44, CH-1011 Lausanne, Switzerland. Phone +41 21 314 47 14, fax +41 21 314 83 60. E-mail: Montserrat.Fraga@chuv.ch, Roland.Sahli@chuv.ch or Darius.Moradpour@chuv.ch

[#]Current affiliation: J. Craig Venter Institute, La Jolla, CA 92037, USA

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Abbreviations: ALT, alanine aminotransferase; HEV, hepatitis E virus; ORF, open reading frame; pb, posterior probability.

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Key points

- We report the largest series to our knowledge of PCR-proven autochthonous acute hepatitis E.
- Middle-aged and elderly men constitute the majority of symptomatic patients, with particularly severe courses in patients with underlying chronic liver disease.
- Most infections were due to hepatitis E virus of genotype 3, with limited clusters identified by phylogenetic analyses; three cases of genotype 4 infection acquired in Switzerland were also documented.
- Testing for HEV should be included early in the diagnostic workup of acute hepatitis and of neuralgic amyotrophy, a typical extrahepatic manifestation of acute hepatitis E of genotype 3.

Abstract

Background: Autochthonous hepatitis E is increasingly recognized as zoonotic infection in western countries. Serologic assays have varying sensitivity and specificity.

Methods: We implemented molecular testing to identify and characterize acute hepatitis E acquired in Switzerland.

Results: Ninety-three cases of mostly symptomatic acute hepatitis E acquired in Switzerland were documented by PCR between November 2011 and December 2016. Median HEV RNA was 7.5×10^4 IU/ml (range, 5.3 to 4.7×10^7 IU/ml). HEV genotyping was successful in 78 patients, revealing genotype 3 in 75 and genotype 4 in 3 patients. Phylogenetic analyses revealed a few limited geographial and temporal clusters. Of the 91 patients with available anti-HEV IgM serology, 4 were negative; three of these were also IgG-negative, likely as a result of immunosuppression, and one was IgG positive, a constellation compatible with HEV reinfection. Median age of the patients was 58 years (range, 20-80 years); 71 (76.3%) were men and 49 of these (69.0%) were ≥ 50 years old. The clinical course was particularly severe in patients with underlying chronic liver disease, with fatal outcome in two patients. Six patients (6.5%) presented with neuralgic amyotrophy.

Conclusions: Nucleic acid-based diagnosis reveals HEV as a relevant cause of acute hepatitis in Switzerland. Middle-aged and elderly men constitute the majority of symptomatic patients. Testing for HEV should be included early in the diagnostic workup of acute hepatitis and of neuralgic amyotrophy, a typical extrahepatic manifestation of HEV genotype 3 infection.

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1 | INTRODUCTION

Hepatitis E virus (HEV) is believed to be the most common cause of acute hepatitis and jaundice in the world.[1, 2] It is a positive-strand RNA virus classified in the *Hepeviridae* family.[3] Two scenarios have been described: On the one hand, HEV genotypes 1 and 2 cause waterborne outbreaks mainly in the developing world, infecting about 20 million people and claiming 70,000 lives every year.[4] On the other hand, HEV genotypes 3 and 4 have been recognized as zoonotic infections in the developed world, with much higher than anticipated seroprevalence rates, reaching 86.4% in the Southwest of France.[5]

HEV genotype 3 has a worldwide distribution while genotype 4 has been found mainly in China and Southeast Asia (refs. 3 and 6 as well as references therein). Both infect humans as well as swine, wild boar, deer and other animal species. Transmission to humans is believed to occur mainly through the consumption of raw or undercooked meat but other routes of transmission have been suggested.[7, 8]

The prevalence of anti-HEV IgG in Switzerland has been reported to range between 4.2 and 21.8%, depending on the serologic assay used.[9, 10] This broad range points to the varying sensitivity and specificity of currently available serologic assays for hepatitis E, as documented in other studies.[11, 12]. In addition, the epidemiological, virological and clinical features of HEV infection acquired in Switzerland have not been described. On this background, we explored molecular testing for HEV infection to characterize acute hepatitis E acquired in Switzerland.

2 | PATIENTS AND METHODS

2.1 | Patients

Molecular testing for HEV at the University Hospital Lausanne has been offered to the Swiss medical community since November 2011. Samples were sent in a context of diagnostic workup for mostly symptomatic hepatitis. Demographic characteristics as well as clinical and laboratory data from all patients with a positive PCR result for HEV RNA were collected. The study was approved by the Ethical Committee of the Canton de Vaud, Switzerland (protocol no. 478/15). The Swiss law and acts on research involving human beings allow performing retrospective studies without consent in exceptional cases. Our study was approved by the Ethical Committee under these circumstances since (i) the interest of research was considered to outweigh the interest of the persons and (ii) obtaining consent was considered disproportionately difficult. Patients with a travel history to a classical endemic area in the preceding 3 months and immunocompromised individuals with chronic hepatitis E were excluded from the present analysis.

The following items were recorded: age and gender, travel history in the 3 months preceding the diagnosis of hepatitis E, history of underlying chronic liver disease or immunosuppression, results of anti-HEV IgM and IgG assays, peak alanine aminotransferase (ALT), presence or absence of jaundice and, in patients with jaundice, peak bilirubin levels. Serologic assays were performed locally in the centers that then sent the samples for molecular testing. Samples from patients whose initial diagnostic workup did not include anti-HEV serology and for whom sufficient material was available (n = 6) and those who were reported to be anti-HEV IgM-negative (n = 5), were (re)tested centrally using the assays from Dia.Pro Diagnostic Bioprobes (Milan, Italy).

2.2 | Quantitative PCR and genotyping

Purification of nucleic acids and reverse transcription were performed as described in the Supplementary Material, followed by real-time polymerase chain reaction according to Jothikumar *et al.*,[13] with the updated primer set and reaction conditions listed in Supplementary Table 1. Each set of RNA extractions was accompanied by a negative water control to monitor contamination. The PCR assay showed excellent sensitivity and specificity as well as performance in a European validation panel, as detailed in the Supplementary Material. The international standard received from the Paul Ehrlich Institute in Langen, Germany (ref. 14; PEI code 6329/10; version 1.0; July 7, 2011) was used to quantify results.

HEV genotype was determined by sequencing of touch down PCR amplicons encompassing the open reading frame (ORF) 2/3 region of the viral genome (Supplementary Table 1). Primer sequences were removed prior to phylogenetic analysis of the resulting 191-195-bp fragments by neighbor-joining tree building with the integrated Geneious version 10.0.5 or superior bioinformatic analysis tool (ref. 15; https://www.geneious.com). The 310 full-length HEV sequences available on September 1, 2016 were retrieved from GenBank and clustered to at least 85% nucleotide homology. The 40 resulting contigs, representing at least 4 genomes each, were used as references in tree building, after trimming to the internal amplicon region defined by primers HEVgenF-H and HEVgenR-A (Supplementary Table 1). Phylogenetic analysis was performed with MrBayes[16] using standard parameters for DNA in the Geneious software. HEV of a subset of patients was also genotyped with a PCR protocol amplifying a 595-bp fragment from the ORF2 region recommended for HEV genotyping (refs. 17 and 18 as well as Supplementary Material).

3 | RESULTS

A total of 1981 samples were sent for diagnostic PCR testing between November 2011 and December 2016, the majority for symptomatic hepatitis, with a continuous increase over the years (Figure 1A). Of these, 145 (7.3%) samples from 111 patients were positive for HEV RNA.

Demographic, clinical and laboratory data was obtained for 110 patients (Figure 1B). Of these, 8 had recently traveled to Asia and were excluded from the present analysis (7 HEV genotype 1 infections acquired in the Indian subcontinent and one HEV genotype 4 infection likely acquired in China). Moreover, we also excluded 9 cases of chronic hepatitis E of which 7 were documented in liver transplant recipients, one in a kidney transplant recipient and one in a bone marrow transplant recipient. Hence, data from 93 patients with acute hepatitis E likely acquired in Switzerland were included in the present analysis (Supplementary Table 2).

Median HEV RNA was 7.5×10^4 IU/ml (range, 5.3 to 4.7×10^7 IU/ml). HEV genotyping with the ORF2/3-based assay was successful in 78 patients, revealing infection with genotype 3 in 75 and with genotype 4 in three. HEV genotyping was unsuccessful in 15 patients of which the majority had low viral titers.

Phylogenetic tree analysis of the identified HEV genotype 3 and 4 sequences is shown in Figure 2A. All nodes to the genotypic levels were supported by a high posterior probability (pb), as denoted by an asterisk (HEV genotype 1: pb = 1; genotype 4: pb = 1; genotype 3: pb = 0.98). Phylogenetic analyses, although not always robust, revealed a few limited geographical and temporal clusters according to Canton of residence (Figure 2B) and year of acute hepatitis E. For example, patients 24, 30 and 32, who were infected with a closely related virus (pb = 0.74), all resided in the Canton Ticino and developed acute hepatitis E within a 6-month interval. As another example, the viral strains isolated from patients 81, 86 and 89 (pb = 1) showed a similar geographical clustering within a 20-km range and a 2-month interval. These clusters were further confirmed with the ORF2-based genotyping assay (data not shown).

Several patients with distinct clinical outcomes were under the same highly supported phylogenetic node (e.g. patients 35 and 43 with neuralgic amyotrophy and death due to acute-on-chronic liver failure, respectively; pb = 1). Conversely, patients presenting with similar clinical outcomes were distributed under different nodes (e.g. patients 8 and 35 as well as 14, 46, 47 and 68 with neuralgic amyotrophy). These data are consistent with a lack of association of specific HEV genotype 3 strains with defined clinical outcomes.

The three genotype 4 infections were all observed in the northeastern part of Switzerland. Seasonal variation of the number of submitted samples or the percentage of positive samples was not evident (data not shown).

Results of anti-HEV IgM testing were available in 91 patients and results of both anti-HEV IgM and IgG in 90. Of the latter, 7 (7.8%) were positive for anti-HEV IgM but negative for IgG, 79 (87.8%) were positive for both anti-HEV IgM as well as IgG, and one was negative for anti-HEV IgM but positive for IgG. Three patients were negative for both anti-HEV IgM and IgG: one had received chemotherapy and rituximab for lymphoma 7 months prior to acute hepatitis E, one was a renal transplant recipient and one was treated with predisone 20 mg per day for pulmonary fibrosis. Hence, of the 91 patients with available anti-HEV IgM serology, 4 were negative. Three of these were also IgG-negative, likely as a result of immunosuppression, and one was IgG positive, a constellation compatible with HEV reinfection.

Seventy-one of the 93 patients (76.3%) were men. Median age of all patients was 58 years, with a range between 20 and 80 years and an interquartile range between 47 and 69 years. Sixty-three of all patients (67.7%) and 49 of male patients (69.0%) were ≥ 50 years old.

Fourty-nine patients (52.7%) presented with jaundice. In this setting, peak total bilirubin ranged between 45 and 602 µmol/l (median 120 µmol/l). In the absence of jaundice, PCR testing for HEV RNA was generally motivated by flu-like symptoms, including myalgia, arthralgia and low-grade fever, or by neurologic symptoms (see below) associated with elevated ALT levels. Median peak ALT was 1378 U/l, with a wide range between 80 and 5001 U/l.

The clinical course was particularly severe in patients with underlying chronic liver disease. Four patients experienced decompensation of pre-existing cirrhosis, with ascites, hepatorenal syndrome and/or hepatic encephalopathy, but ultimately recovered with supportive treatment. However, two patients with underlying cirrhosis developed acute-on-chronic liver failure with a fatal outcome. They were 80 and 74 years old, respectively, presented initially with deep jaundice as well as ascites and subsequently developed renal failure and hepatic encephalopathy. Alcoholic hepatitis was initially suspected in both patients. Ribavirin was started upon documentation of HEV infection, 5 days before death in the first and one day before death in the second patient.

Drug-induced liver injury was initially suspected in at least two patients (liraglutide for diabetes in patient 8 and erlotinib for lung cancer in patient 9; Supplementary Table 2).

Six of the 93 patients (6.5%) presented with bilateral, asymmetric neuralgic amyotrophy (also known as brachial neuritis or Parsonage-Turner syndrome). All of these were men infected with HEV genotype 3. Their median age was 50 years, with a narrow range between 45 and 62 years. Peak ALT levels in these patients ranged from 99 to 2579 U/l; only two developed jaundice. Neurological manifestations have been reported in refs. 19 and 20. One patient was treated with prednisone and ribavirin. The others were treated with corticosteroids and/or intravenous immunoglobulins according to local expertise. The outcome was overall favorable in all patients, with resolution of neuralgic pain. However, minor motor deficits persisted in the majority as per their last control exam performed up to > 2 years after the acute episode.

4 | DISCUSSION

We successfully implemented nucleic acid-based diagnosis of hepatitis E**.** Between November 2011 and December 2016 we observed a steady increase in demand for molecular testing, likely reflecting enhanced awareness for hepatitis E in the Swiss medical community, although a true increase in the incidence of HEV infection and/or the number of symptomatic cases cannot be excluded, as also discussed in a recent study addressing the risk of foodborne HEV transmission in Switzerland.[21] The proportion of positive samples remained roughly constant over the years, allowing us to characterize 93 patients with acute hepatitis E acquired in Switzerland. Samples for molecular testing were sent by physicians across Switzerland mostly in a context of diagnostic workup for symptomatic acute hepatitis. In 2016, one new patient with acute hepatitis E was identified every 9 days, i.e. almost every week.

To the best of our knowledge, this is the largest series so far to evaluate PCR-proven acute hepatitis E. Indeed, most of the published studies are based on serological assays. However, serological tests for anti-HEV IgM and IgG have varying sensitivity and specificity.[10-12] Nijskens *et al*. recently reported 79 patients with HEV genotype 3 infection from the Netherlands.[22] Kokki *et al*. reported 39 patients with PCR-proven acute hepatitis E from Scotland.[23]

It is important to note that some samples sent for testing may have been PCR-negative because they were sent after the window of PCR positivity. In fact, it is currently believed that HEV RNA is cleared from serum or plasma within an average of 3 weeks after the appearance of symptoms.[24, 25] Hence, molecular and serological testing may be regarded as complementary in making a diagnosis of acute hepatitis E, especially if the sample for PCR testing is not addressed early enough.

Our study cannot provide any information on the incidence of hepatitis E in Switzerland. However, based on our data, the facts that hepatitis E is often diagnosed by serology alone and that other laboratories now also offer molecular testing, as well as the notion that most autochthonous HEV infections may occur in an asymptomatic or oligosymptomatic fashion, HEV is likely more frequent than hepatitis A, B or C viruses as a cause of acute hepatitis in Switzerland. Given the central location of Switzerland, our findings may apply also to other European countries.

Interestingly, the majority of patients for whom this information was available did not recall any specific at-risk food consumption. Only a minority indicated the consumption of home-made pork sausage, undercooked pork liver or raw game meat.

Most cases of HEV infection acquired in Switzerland were found to be of genotype 3. However, we also identified three genotype 4 infections in patients who had not travelled to typical endemic regions for this genotype, i.e. China and Southeast Asia. Although scarce, isolated cases of autochthonous HEV genotype 4 infections have been reported in other European countries, including France, Italy, Germany and Denmark.[26-28] The clinical course in our patients with HEV genotype 4 infection was particularly severe in one while the other two had moderate hepatitis without jaundice.

The 191-195-bp fragment of the HEV ORF2/3 region amplified by our PCR assay is sufficient for assignment to each of the 4 main human pathogenic genotypes. However, this assay does not allow for reliable isolate distinction, as indicated by weak pb values (< 0.90) at specific nodes of the tree. A few limited geographial and temporal clusters according to Canton of residence and year of acute hepatitis E are nevertheless evident in our phylogenetic tree analysis. This clustering can be explained by the separation of certain regions of Switzerland, such as the Canton Ticino, by the Alps, a large mountain chain.

ORF2-based genotyping with a larger DNA fragment has been reported to be more appropriate for subtyping.[17, 18] We used a phylogenetic tree comparison method (Compare2Trees; ref. 29) to assess the prediction ability of the ORF2/3-based method described in this work and of the ORF2-based method. Using the reference tree built from the full-length genomes available in Genbank as of May 31, 2017, the ORF2-based method was not significantly superior to the ORF2/3-based method (topological scores of 80.7% and 78.7%, respectively; P = 0.1204). We nevertheless reassessed HEV genotypes with the ORF2-based PCR. This assay was found to be less sensitive in our setting, allowing to successfully analyze the samples from 31 patients and confirming the limited clusters (data not shown). As also suggested by the ORF2/3-based genotyping method, ORF2-based phylogeny did not reveal any association of specific viral strains with defined clinical

outcomes. Of note, both genotyping methods taken together found the cases associated with neuralgic amyotrophy to be dispersed over the branches, with the exception of two cases from the Canton Ticino where geographical localization may explain the clustering. These findings are in line with a previous study which did not find any association of specific HEV genotype 3 variants and clinical manifestations.[30]

Anti-HEV IgM were negative in 4 of 91 patients with available serology. Three of these were also IgG-negative, likely as a result of immunosuppression, and one was IgG positive, possibly reflecting reinfection. We confirm, therefore, that serologic assays are not reliable in the setting of immunosuppression and that molecular testing should be used in this situation.

In line with previous studies,[22, 31, 32] we found that middle-aged or elderly men represent the majority of patients presenting with symptomatic acute hepatitis E. Indeed, $^{3}/_{4}$ of our patients were men and more than $^{2}/_{3}$ of these were ≥ 50 years old. The reasons for this particular susceptibility remain currently unknown.

Most patients in our series experienced self-limited hepatitis with a favorable outcome. However, HEV infection can cause significant morbidity and non-negligible mortality in patients with pre-existing chronic liver disease or due to extrahepatic, notably neurologic complications. A particularly severe course has been described in patients with underlying cirrhosis.[22, 31, 33, 34] Two of the 93 patients (2.2%) in our series died as a consequence of acute HEV infection. Both had underlying cirrhosis and an initial diagnosis of alcoholic hepatitis had been made, motivating corticosteroid treatment in one. Ribavirin treatment was introduced in both patients once HEV infection was documented. However, the patients died shortly thereafter. Earlier diagnosis may have allowed to initiate ribavirin treatment more rapidly and successfully, as described in recent case series.[35-37] Thus, testing for HEV infection should be considered in patients with presumed alcoholic hepatitis.

Interestingly, several patients in our series spontaneously resolved acute hepatitis E despite immunosuppressive drug treatment, including 6 who were on corticosteroids. Only one of the latter had received high-dose corticosteroids in the course of acute hepatitis E while the others were treated with prednis(ol)one at doses of 10 to 20 mg per day. Hence, it appears, based on these few patients, that corticosteroids alone at low to moderate doses did not interfere with the patients' ability to eliminate HEV.

Drug-induced liver injury is characterized by a wide range of both phenotype and severity. Diagnosis can be challenging and is generally based on chronological association between incriminated drug administration and liver injury. As previously reported,[38, 39] acute hepatitis E was initially misinterpreted as drug-induced liver injury in at least two patients in our series.

Therefore, hepatitis E should be included in the differential diagnosis of drug-induced liver injury.

Neurologic symptoms have been reported in retrospective studies in up to 5% of patients with HEV genotype 3 infection (reviewed in ref. 40). The hallmark neurological complication is neuralgic amyotrophy, and both immunological mechanisms as well as direct neurotropism have been discussed. In our study, 6 of 93 patients (6.5%) presented with neuralgic amyotrophy. They were men between 45 and 62 years (median 50 years) infected with genotype 3. Neurological manifestations have been reported in refs. 19 and 20. Most patients were treated with corticosteroids and/or intravenous immunoglobulines, according to local expertise. One patient was treated with prednisone and ribavirin 1000 mg per day, with negativation of HEV RNA within one week. Ribavirin treatment was pursued for 2 weeks after negativation of HEV RNA in plasma and stool, i.e. for a total of 3 weeks. Neuralgic pain resolved and muscle strength in the shoulder girdle improved rapidly; however, right-sided diaphragmatic paralysis persisted. Ribavirin treatment in the context of HEV-associated neuralgic amyotrophy requires further study.

An inherent limitation of our study is the retrospective data collection. However, we were able to reach almost all practitioners throughout Switzerland to obtain demographic, clinical and laboratory data, and missing data were scarce. On the other hand, the multicentric data collection constitutes a strength of the study, reflecting "real-life" clinical experience with a majority of immune competent symptomatic patients. In addition, we restricted our study to PCR-documented cases of acute hepatitis E, thereby eliminating potential limitations related to the sensitivity and specificity of serological diagnosis.

In conclusion, nucleic acid-based diagnosis reveals HEV as a relevant cause of acute hepatitis in Switzerland. Middle-aged and elderly men constitute the majority of symptomatic patients. Testing for HEV should be included early in the diagnostic workup of acute hepatitis and of neuralgic amyotrophy, a typical extrahepatic manifestation of HEV genotype 3 infection.

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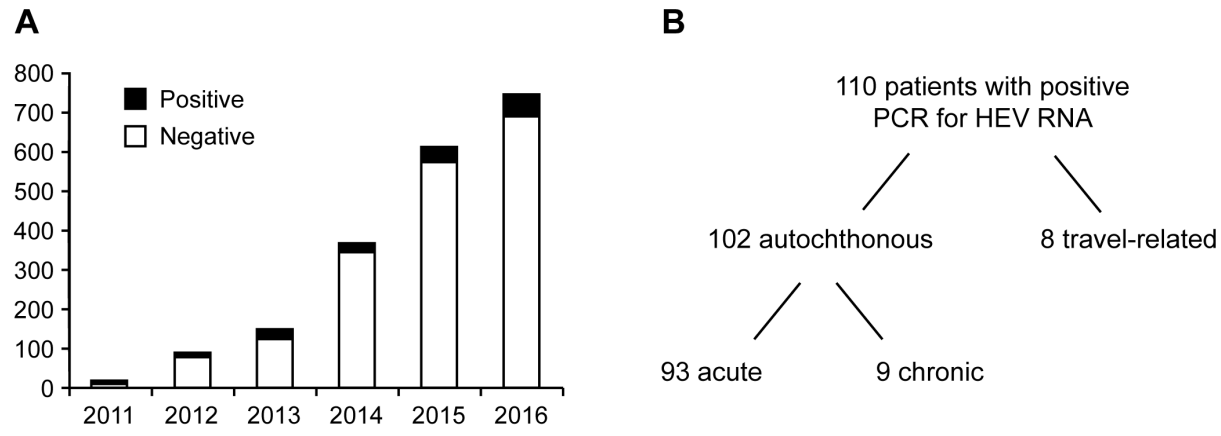
LEGENDS TO FIGURES

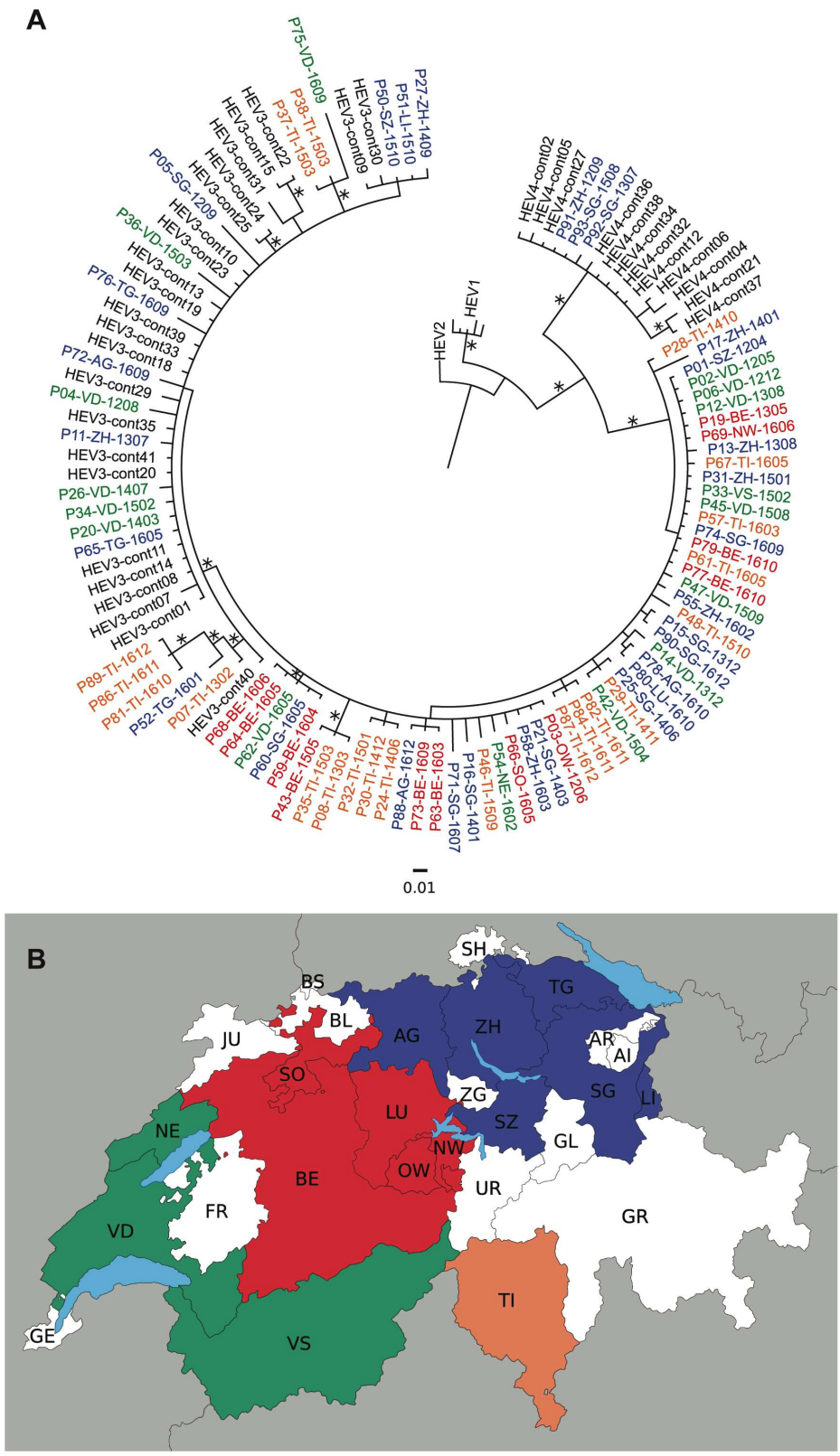
FIG. 1. Analyses performed and patient disposition. (A) Number of HEV RNA analyses performed between 2011 and 2016. The number of positive samples is indicated in black. A total of 1981 samples were analyzed between 01.11.2011 and 31.12.2016, with 145 (7.3%) positive samples from 111 patients. **(B)** Disposition of the 110 patients with positive HEV RNA for whom clinical information was available.

FIG. 2. Phylogenetic tree analysis. (A) Phylogenetic tree of 78 patient samples which could be successfully genotyped based on the 191-195-bp fragment of the HEV open reading frame 2/3 region and of 40 contig sequences representing the 310 full-length HEV genomes available in GenBank in September 2016. HEV genotype 2 was arbitrarily selected as outgroup. Nodes with a high posterior probability value (> 0.95) are indicated by an asterisk. Each sample is denoted by patient number, Canton of residence (using the same color code as in panel B) as well as year and month. The scale bar indicates the average number of substitutions per column of the alignment. **(B)** Map of Switzerland. The map was prepared using the tools available at http://d-maps.com. Samples from patients living in the following Cantons have been analyzed: AG, Aargau; BE, Berne; LU, Lucerne; NE, Neuchâtel; NW, Nidwalden; OW, Obwalden; SG, St. Gallen; SO, Solothurn; SZ, Schwyz; TI, Ticino; TG, Thurgau; VD, Vaud; VS, Valais; ZH, Zurich. One sample originated from Liechtenstein (LI).



Fraga *et al*.

Figure 1



Fraga *et al*.

Figure 2

SUPPLEMENTARY MATERIAL

Supplementary Methods

Quantitative PCR. Total nucleic acids were purified from 200 µl plasma or serum supplemented with an internal RNA control with the MagNaPure 96 Total Nucleic Acids kit according to the manufacturer (Roche, Basel, Switzerland) and eluted in 100 µl elution buffer. Ten µl of the eluate was converted to cDNA in a 20-µl reaction (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA), adjusted to 50 µl with PCR grade water, and 5 µl was subjected to real-time polymerase chain reaction according to Jothikumar N *et al*. (J Virol Methods 2006;131:65-71) with the updated primer set and reaction conditions listed in Supplementary Table 1. Each set of RNA extractions was accompanied by a negative water control to monitor contamination.

Reaction conditions. For real-time PCR, the primers were ordered separately (Eurogentec, Seraing, Belgium) and mixed to 10 µM each as forward and reverse stocks in PCR grade water. The probe was also adjusted to 10 µM. The forward and reverse primers and the probe were then added at 200 nM each in a real-time PCR reaction mixture containing 10 µl 2x TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µl cDNA and PCR grade water to 20 µM final volume. Real-time PCR was performed in ABI instruments (ABI7900 and Quantstudio VI7) with the following cycling profile: 9 min at 95°C, followed by 50 cycles of 95°C for 1 sec and 60°C for 20 sec. HEV-negative results were valid only if the RNA internal control was positive at a similar Cp (crossing point) to the water-only extraction control.

For ORF2/3-based genotyping, 2 µl cDNA was amplified with Amplitaq Gold (Applied Biosystems; 0.625 units) in a 25 µl mixture containing 1x Amplitaq Gold buffer, 3 mM $MgCl_2$ and 100 nM of each primer. The PCR profile was 95°C for 8 min, followed by 20 cycles of touch down PCR (94°C for 30 sec, touch down from 57°C to 47°C at 0.5°C reduction steps for 30 sec each, 72°C for 30 sec), followed by 25 cycles of conventional PCR (94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec) and a final 5 min step at 72°C. Five µl PCR reaction was analyzed by gel electrophoresis. Positives were sequenced (each PCR primer) with Big Dye terminator chemistry and resolved on a 3130 XL instrument (Applied Biosystems). Sequences were then mapped against the HEV reference sequence. When the viral load was < 50,000 copies/ml plasma, it was preferable to treat the nucleic acid preparation with DNAse (Ambion, Thermo Fisher Scientific, Waltham, MA) prior to cDNA synthesis for successful genotyping PCR amplification.

For ORF2-based genotyping, 2 µl cDNA was amplified with Amplitaq Gold Fast PCR Master Mix kit (Applied Biosystems) in a 25 µl mixture with either HEV-3156rs or HEV-3162rs (to optimize coverage) and HEV-3159rs primers each at 800 nM according to the manufacturer. The PCR profile was 95°C for 8 min, followed by 20 cycles of touch down PCR (94°C for 30 sec, touch down from 60°C to 50°C at 0.5°C reduction steps for 30 sec each, 72°C for 30 sec), followed by 25 cycles of conventional PCR (94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec) and a final 5 min step at 72°C. Positives were sequenced as described for ORF2/3-based genotyping.

Validation of the real-time PCR assay. As stated above, the real-time PCR assay was adapted from an already validated method described by Jothikumar N *et al*. (J Virol Methods 2006;131:65-71), thus requiring less extensive validation. Analytical specificity was 100% using samples from patients presenting with hepatitis A, B, C or delta, as well as with human immunodeficiency virus. Sensitivity was assessed with cDNA positive controls; all reactions were positive with 100,000 and 1000 copies per reaction (n = 524 each) and 91% were positive with the 10 copies (n = 1060). Ten copies in a reaction translate to 375 HEV international units (IU) per ml of plasma. The average slope of the amplification plots (n = 524) was −3.56 (PCR efficiency of 91%). Participation in the Quality Control for Molecular Diagnosis (http://www.qcmd.org) from 2013 to 2016 was successful for all core

samples (n = 24, mostly genotype 3) and in 5 of 8 educational samples containing low viral loads close to our limit of detection of 375 IU/ml of plasma.

Supplementary Table 1. Primers and probes used for molecular assays.

	Name	Sequence (5'-3')
Forward primers for real-time PCR	JVRSHEV_F1 (5310)	CGGTGGTTTCTGGGGTGAC
	JVRSHEV_F2	GCAGTGGTTTCTGGGGTGAC
	JVRSHEV_F3	GCGGTGGTTTCTGGAGTGAC
	JVRSHEV_F4	GCGGTAGTTTCTGGGGTGAC
Reverse primers for real-time PCR	JVRSHEV_R1	CAAAGGGGTTGGTTGGATGAA
	JVRSHEV_R2 (5382)	GAAGGGGTTGGTTGGATGAA
	JVRSHEV_R3	CGAAAGGGTTGGTTGGATGAA
	JVRSHEV_R4	AAGGGGTTGGCTGGATGAA
	JVRSHEV_R5	CGAAGGGATTGGTTGGATGAA
Probe for real-time PCR	JVRSHEV_Pt (5336)	FAM-ATTCTCAG+C+C+CYTYGC-BHQ1
ORF2/3 forward primer for genotyping	HEVgenF-H (5152)	GGGTGGAATGAATAACATGT
ORF2/3 reverse primer for genotyping	HEVgenR-A (5379)	GGGGTTGGTTGGATGAA
ORF2 forward primer for genotyping	HEV-3156rs (5734)	TCCAATTATGCCCAGTATCGGGT
Alternative ORF2 forward primer for genotyping	HEV-3162rs (5638)	GCTTCGGGTACTAATTTGGT
ORF2 reverse primer for genotyping	HEV-3159rs (6368)	ACAGAATTGATTTCGTCGGC

Numbers in parentheses indicate the position of the 5’ end relative to the reference sequence (GenBank accession number AF060669).

+C denotes locked nucleic acids used to increase the Tm of the probe. BHQ, black hole quencher; FAM, 6-carboxyfluorescein.

Supplementary Table 2. Clinical characteristics of 93 patients with acute hepatitis E documented by PCR for HEV RNA.

Pat.	Sex (m/f)	Age (yrs)	HEV RNA (IU/ml)	Geno-type	Cirrhosis (y/n)	Immune suppr. (y/n)	NA (y/n)	Peak ALT (U/l)	Jaundice (y/n)*	anti-HEV IgM	anti-HEV IgG	Comments
1	m	69	2.6E+04	3	n	n	n	1238	n	pos	pos	
2	f	56	1.2E+06	3	n	n	n	1322	y (187)	pos	neg	
3	m	64	6.2E+03	3	n	n	n	n.a.	y (141)	pos	pos	
4	m	75	8.3E+03	3	n	y	n	1200	y (69)	pos	pos	Prednisolone 15 mg qd for RA
5	m	71	5.2E+04	3	n	n	n	793	y	pos	pos	
6	m	72	5.6E+04	3	n	n	n	260	n	pos	pos	
7	m	56	3.9E+06	3	n	n	n	2000	n	pos	pos	
8	m	60	2.0E+05	3	y	n	y	1500	y (406)	pos	pos	
9	m	67	2.6E+03	n.a.	y	y	n	950	y	pos	pos	Erlotinib for lung cancer
10	f	58	1.3E+03	n.a.	n	y	n	n.a.	n	pos	pos	High-dose corticosteroids for Still disease
11	m	46	6.3E+01	3	n	n	n	400	n	pos	pos	
12	f	37	5.6E+03	3	n	y	n	n.a.	n	pos	pos	Pulmonary transplant recipient
13	m	33	1.5E+05	3	n	n	n	1486	y (190)	pos	pos	
14	m	52	4.4E+05	3	n	n	y	2579	n	pos	neg	
15	m	42	7.5E+05	3	n	n	n	3500	y (122)	pos	pos	
16	m	46	8.3E+04	3	n	n	n	4660	y (244)	pos	pos	
17	m	41	2.1E+07	3	n	y	n	3403	n	neg	neg	Lymphoma treated with R-CHOP 7 months earlier
18	m	71	5.3E+01	n.a.	n	n	n	663	n	pos	pos	
19	m	53	2.2E+05	3	n	n	n	3552	y (157)	n.a.	n.a.	
20	m	50	9.8E+03	3	n	n	n	753	n	pos	pos	
21	m	73	4.2E+03	3	y	y	n	344	y (76)	pos	pos	Rituximab for lymphoma
22	m	73	7.5E+02	n.a.	n	n	n	750	y (77)	pos	pos	
23	m	72	5.3E+00	n.a.	n	y	n	n.a.	y (228)	neg	neg	Prednisone 20 mg qd for pulmonary fibrosis
24	f	41	4.0E+04	3	n	n	n	500	n	pos	pos	
25	m	44	1.7E+06	3	n	n	n	2549	n	pos	n.a.	
26	m	29	1.1E+04	3	n	n	n	232	n	pos	pos	
27	m	52	7.0E+03	3	n	n	n	n.a.	y (150)	pos	pos	
28	m	42	7.5E+04	3	n	n	n	2000	y (80)	pos	pos	
29	f	31	9.0E+04	3	n	n	n	1734	n	pos	pos	
30	m	67	2.5E+05	3	n	n	n	n.a.	n	pos	pos	
31	m	44	5.6E+04	3	n	n	n	510	y	pos	pos	
32	m	55	1.3E+04	3	n	n	n	n.a.	n	pos	pos	Severe myalgia
33	m	52	3.5E+07	3	n	n	n	n.a.	y	pos	pos	Severe myalgia
34	f	51	1.6E+05	3	n	n	n	1825	n	pos	pos	
35	m	62	2.8E+04	3	n	n	y	n.a.	n	pos	pos	
36	m	46	4.8E+05	3	n	n	n	3193	n	pos	pos	
37	m	65	1.1E+04	3	n	y	n	664	n	pos	pos	Methotrexate for RA
38	m	64	3.5E+05	3	n	n	n	1270	n	pos	pos	
39	f	71	4.8E+05	n.a.	n	n	n	984	n	pos	pos	
40	f	67	2.9E+03	3	n	n	n	n.a.	n	pos	pos	
41	f	46	2.5E+04	n.a.	n	n	n	537	y	pos	pos	Severe myalgia

42	m	50	5.0E+05	3	n	n	n	3028	y (79)	pos	pos	
43	m	80	2.2E+03	3	y	n	n	2600	y (602)	pos	pos	Acute-on-chronic liver failure
44	f	67	6.8E+03	n.a.	n	y	n	700	y	pos	pos	Methotrexate for RA
45	f	80	9.0E+04	3	n	n	n	1215	y (195)	pos	pos	
46	m	62	9.8E+06	3	n	n	y	2462	y (69)	pos	pos	
47	m	45	2.0E+04	3	n	n	y	99	n	pos	pos	
48	f	37	4.5E+04	3	n	n	n	900	y (76)	pos	pos	
49	m	49	9.8E+04	n.a.	n	n	n	1560	n	pos	pos	
50	m	43	4.8E+05	3	n	n	n	4000	y (90)	pos	pos	
51	m	40	6.1E+05	3	n	n	n	1300	y (60)	pos	pos	
52	f	52	2.3E+05	3	n	y	n	1656	y (170)	pos	pos	Prednisolone for Takayasu arteriitis
53	m	74	8.3E+03	n.a.	y	n	n	705	y (458)	pos	pos	Acute-on-chronic liver failure
54	m	48	3.9E+04	3	n	n	n	1800	y (77)	pos	pos	
55	m	45	3.3E+04	3	n	n	n	2500	y (160)	pos	pos	
56	f	48	2.4E+04	n.a.	n	n	n	947	n	pos	pos	
57	m	75	9.0E+04	3	n	n	n	1445	y (196)	pos	pos	
58	f	50	1.1E+06	3	n	y	n	1054	n	pos	pos	Leflunomide and prednisolone for SLE
59	m	75	1.5E+05	3	n	n	n	3353	y (71)	pos	neg	
60	m	59	2.7E+06	3	n	n	n	1882	n	pos	neg	
61	m	63	2.3E+05	3	n	y	n	600	n	pos	pos	Renal transplant recipient
62	m	20	1.5E+04	3	n	n	n	4000	y (270)	pos	pos	
63	f	75	3.7E+04	3	n	n	n	3463	y (46)	pos	pos	
64	m	72	4.8E+05	3	y	n	n	809	y (189)	pos	pos	
65	m	62	7.5E+04	3	n	n	n	440	y	pos	pos	
66	f	64	9.8E+04	3	n	y	n	n.a.	y	pos	pos	Osteomyelofibrosis
67	m	45	1.7E+05	3	n	n	n	5001	y (45)	pos	pos	
68	m	49	1.1E+05	3	n	n	y	1111	n	pos	pos	
69	m	73	6.5E+05	3	n	n	n	1500	y (97)	pos	pos	
70	m	57	1.7E+03	n.a.	n	n	n	1947	n	pos	pos	Severe myalgia
71	m	71	3.6E+05	3	n	n	n	488	y (162)	pos	pos	
72	m	61	4.2E+05	3	n	n	n	1849	n	pos	pos	
73	m	67	2.2E+04	3	n	n	n	1404	y (93)	pos	pos	
74	m	33	9.8E+04	n.a.	n	n	n	1110	y (46)	n.a.	n.a.	
75	m	72	1.1E+07	3	n	y	n	478	n	pos	pos	Renal transplant recipient
76	m	71	7.4E+04	3	n	y	n	1405	y (134)	pos	pos	Chronic lymphocytic leukemia
77	m	62	1.7E+04	3	n	n	n	2192	n	pos	pos	
78	m	88	7.5E+05	3	n	y	n	1500	n	pos	pos	Methylprednisolone and mycophenolate for minimal change disease
79	m	49	3.8E+04	3	n	n	n	2000	y (101)	pos	pos	
80	f	47	1.5E+04	3	n	n	n	1672	n	pos	pos	
81	m	46	2.2E+07	3	n	n	n	1100	y (55)	pos	neg	
82	f	54	4.7E+07	3	n	y	n	521	n	neg	neg	Renal transplant recipient
83	f	47	1.4E+03	n.a.	n	y	n	805	n	pos	neg	Chemotherapy for breast cancer
84	m	74	5.3E+06	3	n	n	n	1500	y (120)	pos	pos	
85	f	53	1.1E+04	n.a.	n	n	n	390	n	pos	pos	Severe myalgia

86	m	76	2.3E+05	3	n	n	n	2985	y (86)	pos	pos	
87	m	53	2.2E+04	3	n	n	n	1352	n	pos	pos	
88	f	63	2.9E+05	3	n	n	n	1476	n	pos	pos	
89	m	77	1.4E+06	3	n	n	n	800	n	pos	pos	
90	m	64	2.8E+06	3	n	n	n	2456	y (72)	pos	neg	
91	m	59	5.2E+04	4	n	n	n	2450	y (256)	pos	pos	Severe course in the absence of pre-existing cirrhosis
92	m	62	1.2E+03	4	n	n	n	80	n	pos	pos	
93	m	62	4.6E+02	4	n	n	n	591	n	neg	pos	

*Peak total bilirubin levels in patients with jaundice are given in parentheses in µmol/l.

ALT, alanine aminotransferase; HEV, hepatitis E virus; n, no; n.a., not available; NA, neuralgic amyotrophy; neg, negative; Pat., patient; pos, positive; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; y, yes.